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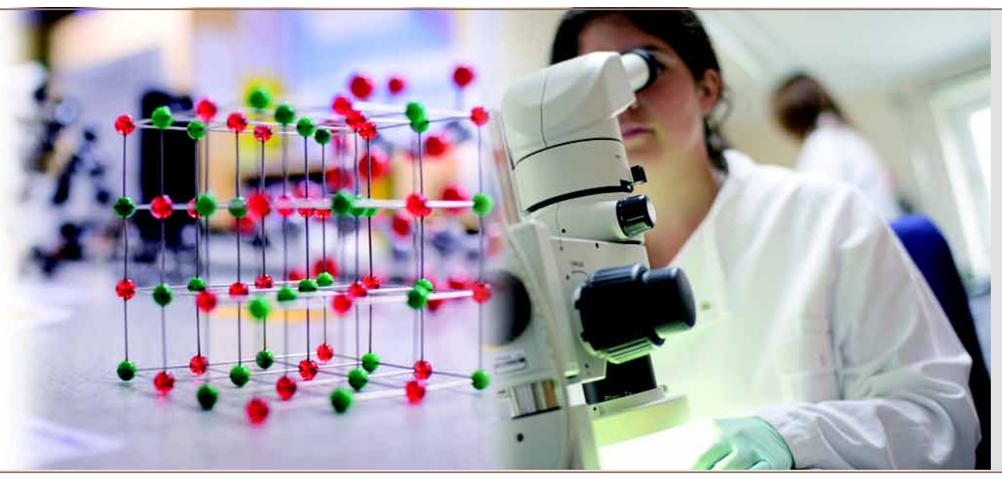

Introduction of a New Reconstructed Human Epidermis with Integrated Functional Primary Melanocytes

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Introduction

The development of novel cosmetics for skin tanning or bleaching is an important issue for cosmetic and chemical industry. The active ingredients used in these products have to be tested for efficacy and product safety. Here we present a new reconstructed epidermis with integrated melanocytes which is based on the already established technology of Epidermal Skin Test 1000 (EST1000, CellSystems, Germany). We tested different ratios of melanocytes and keratinocytes for the production of skin models and used melanocytes from different ethnic groups.

The newly developed epidermal model was characterized by histology to investigate the structural properties of the epidermis. The localisation of melanocytes was analysed by immunohistochemical staining using anti-HMB45 antibodies. Tyrosinase activity of active melanocytes was detected *in situ* by L-DOPA staining of histological sections.

An important feature for testing effects on skin pigmentation is the feasibility of cultivating the models for at least ten days after substance application without loss of viability. Therefore, the models were cultivated for different periods at the air liquid interphase followed by a standard MTT assay to determine the viability. Finally the melanin content for each approach was measured.

Materials & Methods

Viability

The viability of the epidermis models was determined by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl blue; CAS number 298-93-1)] reduction. The epidermis models were incubated in 300 µL of MTT medium (1 mg/mL) for 3 hours. The precipitated blue formazan product was then extracted with isopropanol. The concentration of formazan was measured by determining the OD at 550 nm.

Melanin content

The melanin was extracted from the epidermis models by incubation in 360 µL of Solvable™ (Perkin Elmer, #6NE9100) at 100 °C. The melanin content was measured by the absorption at 492 nm.

Immunohistology

The epidermis models were fixed in Bouin´s solution. Cryosections of 6 µm were prepared and the melanocytes were labelled with mouse monoclonal anti-HMB45 antibodies (DAKO). The staining was performed using peroxidase conjugated anti-mouse as secondary antibodies with AEC as substrate.

L-DOPA staining

Cryostat sections were mounted on glass slides and fixed for 15 min in 4 % Paraformaldehyde. After washing with PBS the slides were transferred into PBS containing 0.1 % L-DOPA. The negative control was incubated in PBS. The staining reaction was performed for 8 h at room temperature.

Results

Viability

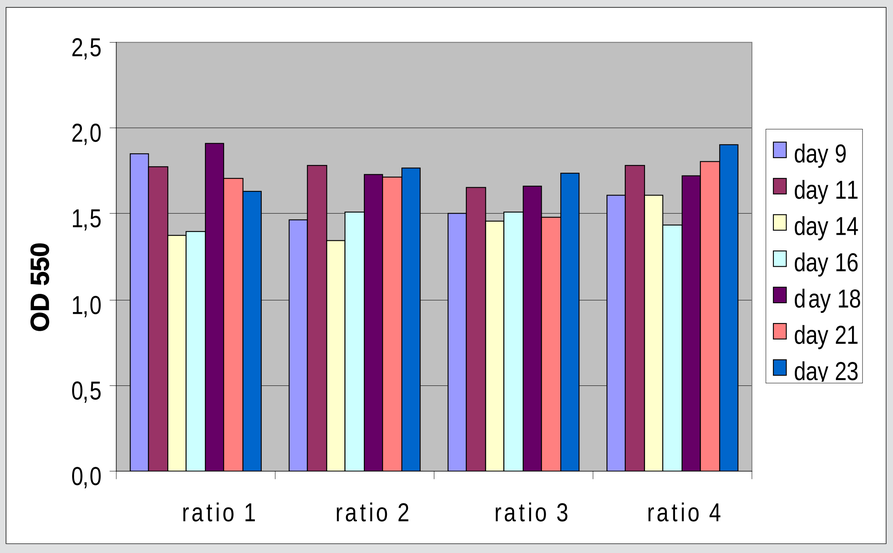


Figure 1: Viability of epidermis models with different keratinocyte-melanocyte ratios after different periods of culture at air-liquid interphase
No significant differences were found from day 9 to day 23 and for all different ratios as determined by MTT-test.

Melanin content

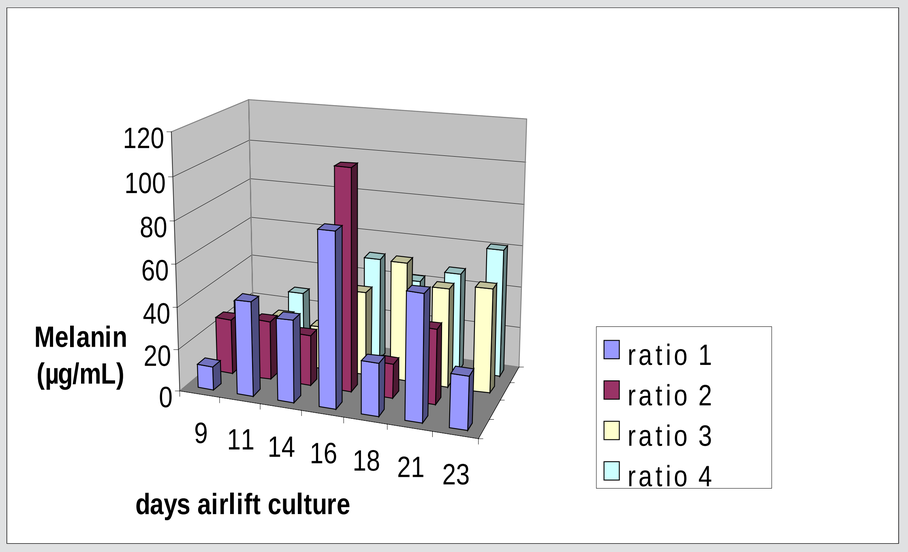


Figure 2: Melanin content of epidermis models with different keratinocyte-melanocyte ratios after different periods of cultivation at air-liquid interphase. A maximum was found for almost all ratios at day 16.

Immunohistochemistry

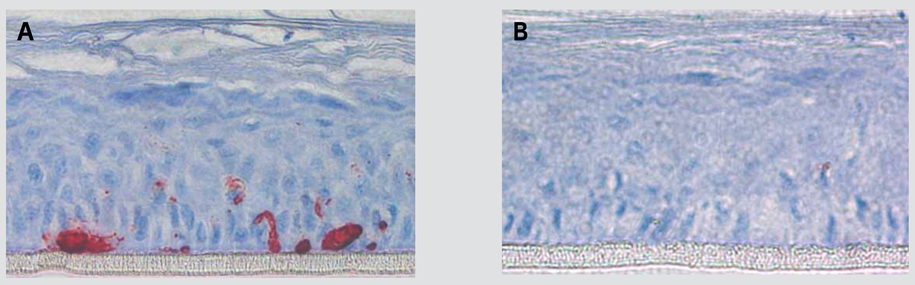


Figure 3: Localisation of melanocytes in the basal layer of the epidermis model using the monoclonal anti-HMB45 antibody. Representative photographs shown. 21 days cultivation at air-liquid interphase. A: anti-HMB45 staining. B: Negative control: isotype antibodies. Counter stain with haematoxylin.

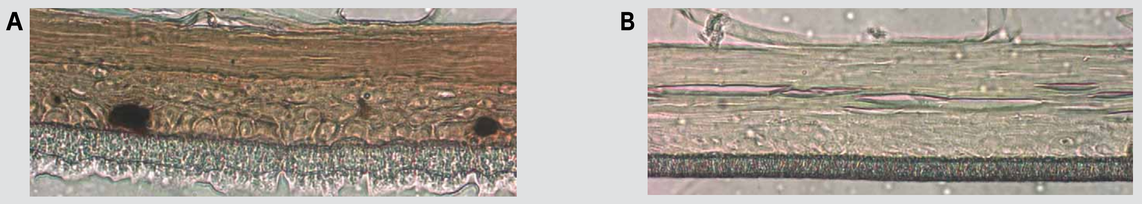
Frequency of melanocytes

Table 1: Number of keratinocytes between two melanocytes in the basal layer. The sections from epidermis models which were cultivated for 9 to 23 days at air-liquid interphase were stained immunohistochemically. The melanocytes were labelled by anti-HMB45 antibodies, counter stained with haematoxylin. The keratinocytes in between two melanocytes in the focal plane were counted. The mean value of 5 analyses is given in the table (n.d. = not determined).

ratio	day 9	day 11	day 14	day 16	day 18	day 21	day 23	mean
1	5,2	4,5	3,0	6,2	4,6	5,3	6,5	**5,0**
2	n.d.	15,0	10,6	11,3	11,0	n.d.	8,0	**11,2**
3	12,0	12,7	11,8	14,0	n.d.	13,0	10,2	**12,3**

L-DOPA staining

Figure 4: Detection of tyrosinase activity by L-DOPA staining. Models were cultivated for 18 days at the air-liquid interphase. A: Cells localized in the basal layer showing positive L-DOPA staining. B: Parallel section treated with PBS as negative control.



Conclusion

The reconstructed epidermis **EST**1000 was established for testing and classification of corrosive and irritating chemicals according to current test guidelines. We upgraded the architecture of **EST**1000 by integrating melanocytes in the basal layer resulting in a different model for different applications. This melanocyte containing epidermal model shows a high comparability to native human skin and additionally the typical distribution of a normal number of melanocytes which show tyrosinase activity as demonstrated by L-DOPA staining. Furthermore we proved that the melanin content of the model could be optimised by varying the ratio of keratinocytes/melanocytes and the cultivation time at the air-liquid interphase. This epidermis model can be cultivated for at least three weeks and allows short term as well as long term studies.

We conclude from these results that the newly developed melanocyte containing epidermal model is a useful tool for research and for characterisation of skin tanning or bleaching substances and for cognate applications which require model systems with melanocytes.